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(54) Title: INCREASED PRODUCTION OF INTERFERON-α

(57) Abstract: The present invention relates to the use of Interferon Regulatory Factor 7 (IRF-7) and functional analogs thereof to modulate the production of type I interferon genes, especially interferon α (IFN-α) genes; INF-7 for use in therapy and pharmaceutical formulations containing the same.

INCREASED PRODUCTION OF INTERFERON - α

The present invention relates to the finding that the Interferon Regulatory Factor 7 (IRF-7) is a critical determinant for the induction of type I interferon genes, especially interferon α (IFN-α) genes. The production of IFN-α is dependent on the presence of IRF-7 and it follows that the production of IFN-α can therefore be enhanced by increasing the levels of IRF-7. The present invention therefore relates to a method of enhancing the production of IFN-α both *in vivo*, and *in vitro*, enhanced expression systems for the production of α-interferon and methods of treatment.

In eukaryotic cells, infection with a virus leads to the induction of the so-called early inflammatory genes. These encode a group of proteins, collectively known as cytokines and chemokines, which are involved in activation of the immune system; as a result, cells such as cytotoxic T cells and other cells recognise the virus- infected cells. The cytokines include a group, the interferons, which also have direct antiviral and antiproliferative properties.

Interferons are classified into two Types, I and II. In man, Type I interferons are the products of transcription of the 13 functional genes of the interferon alpha (IFN-α) family, or of the single interferon beta (IFN-β) or the single interferon omega (IFN-ω) genes. All these genes are clustered on human chromosome 9. Human Type II interferon (interferon gamma, IFN-γ) is encoded by the single IFN-γ gene localized on human chromosome 10.

To produce its effects, an interferon must first bind to a receptor on the cell surface. There are specific receptors of two types, one recognising only IFN-γ, and the other the various Type I interferons. When an interferon binds to a receptor, this triggers an intracellular signalling pathway which leads to activation of a number of genes, collectively termed the "interferon-stimulated genes" (ISG). Some of these encode proteins that can inhibit viral replication; others affect the cell mitotic cycle, or modulate apoptosis; and some have other or as yet unknown functions. Type I interferons in general have direct antiviral effects that are greater than those of IFN-γ, but this latter is a more effective immune modulator.

Type I interferons are in general not produced constitutively, but induced under various circumstances, and in particular when a virus infects cells. In man, production of IFN-α seems normally to be limited to cells of lymphoid origin and epithelial cells, whereas IFN-β can be induced in a large variety of cell types. Recently, it has become clear that for the Type I interferon genes to be expressed, a stimulus is needed that results from the combined action of various cellular factors such as Interferon Regulatory Factors (IRF's). Two members of the IRF family IRF-1 and IRF-2 were originally cloned by their ability to bind to the oligodeoxynucleotide repeats corresponding to the positive regulatory domain (PRDI) in the IFN-β gene promoter. It was shown that IRF-1 is a trans activator which can stimulate transcriptional activity of IFN-α and IFN-β promoters, while IRF-2 which binds to the same oligodeoxynucleotide serves in the same assay as a repressor. It was proposed that in infected cells transcription of IFN-α and IFN-β gene expression was mediated by binding of IRF-1 while in the uninfected cells IRF-2 binds to the promoters of IFN genes and represses the transcription of interferon genes. The critical role of IRF-1 in the induction of IFN-α and -β genes was however eliminated by observation that the knock out mice that had homozygous deletion of IRF-1 were still able to induce both types of interferons. Another factor, named IRF-3 was represented by a single gene and encoded a novel protein of about 50kDa. The expression of IRF-3 was observed in all human tissues and cells examined and was not further stimulated either by viral infection or by IFN treatment. We and others have however found that in transient transfection assay, over-expression of IRF-3 strongly enhances transcriptional activity of both IFN-α and IFN-β promoters in infected cells. It was further shown that in infected cells IRF-3 is post transcriptionaly modified by phosphorylation on serines 385, 386, 396 and 398. Phosphorylation was shown to stimulate transport of IRF-3 into the nucleus and its association with CBP/p300 transcriptional coactivator and was shown to be required for the IRF-3 mediated transcriptional activation: mutants of IRF-3 that were not able to be phophorylated were transcriptionally inactive. IRF-3 is constitutively expressed in a large variety of cells and tissues, and can stimulate expression of IFN-α and IFN-β genes in infected cells; it also plays a critical part in the inducible expression of the RANTES gene. It was further shown that in cells infected with a virus, IRF-3 is phosphorylated; this results in its transport

from the cytoplasm into the nucleus, where it associates with the transcription co-factor, CBP/p300 (Liu et al. (1999) Mol. Cell. Biol. 18 2986 - 2996; Yoneyama et al. (1998) EMBO J 17 1007 - 1095).

We have now found that a further IRF, namely IRF-7 plays a role in the induction of type I interferon genes, particularly IFN-α genes. The human IRF-7, is expressed in lymphoid cells and its expression is enhanced in cells treated with interferon. In infected cells, IRF-7 is transported from cytoplasm to the nucleus, just as is IRF-3. Both human and mouse IRF-7 molecules are able to activate the transcription of interferon alpha gene promoters.

We have found that if a virus infection is to lead to induction of the IFN-β gene, the cell must express IRF-3, as for example, fibroblasts do. Human IFN-α genes on the other hand are induced only in cells such as those of lymphoid origin that express IRF-7 constitutively, or in cells in which formation of IRF-7 has been induced: we have found that human cells produce no IFN-α in the absence of IRF-7. It has been shown that treatment with exogenous IFN-α before infection with a virus efficiently raised the levels of IRF-7 in certain types of cells, so that these could now be induced to yield IFN-α.

We looked at three Namalwa cell lines : two of them were identified as high producers and one as low producer. Each of the high producers produced in our induction experiments about a couple of thousands of units/ml of biologically active IFN (1.7 million of cells/ml/24 hrs) upon induction with Sendai virus ; treatment with butyrate (1.5mM/48 hrs) increased the production to about 15-20 thousands of u/ml (measured by the antiviral activity). In contrast the low producers produced only about 100-200 u/m of interferon and after the butyrate treatment the production increased to only about 1000 u/ml.

We have therefore examined whether the difference in the inducibility between high and low producers of Namalwa cell lines could be due to the difference in relative levels of IRF-3 and/or IRF-7 factors in these cells. There was no significant difference in the relative levels of IRF-3 in high and low producing Namalwa clones as determined by Western hybridization with the rabbit anti IRF-3 serum. The relative levels of IRF-3 decreased at 10-16 hrs post infection which

is in agreement with our previous finding that the phosphorylated IRF-3 is targeted for degradation. In contrast to IRF-3, preliminary results showed there was a difference in the relative levels of IRF-7 between low and high producing Namalwa lines. While in uninfected high producers IRF-7 was expressed at the levels comparable to IRF-3, no (or very low), constitutive expression of IRF-7 was detected in low IFN-α producers. In these low producers IRF-7 was detected only post infection. These results suggest that IRF-7 expression can be stimulated both by viral infection and IFN treatment which in turn suggests that IRF-7 is not expressed constitutively in low producers but its expression can be induced by IFN or by virus. However, when IFN yields from other Namalwa clones said to differ in their yields of IFN-α were checked no significant difference in IFN-α production or IRF-7 levels were in fact found.

Considering the difference between the levels of IRF-7 in the low and high producers as well as the fact that IRF-7 expression is induced by interferon we analyzed and compared the constitutive IFN production in the high and low producers. Our analyses have shown that two high producing Namalwa cell lines produced about 20 u/ml of biologically active interferon constitutively while the low producing line produced less than 2 u/ml of constitutive IFN.

Thus these data indicate that the ability of Namalwa cells to induce synthesis of IFN-α proteins may be determined by the relative levels of IRF-7 in these cells and that the relative levels of IRF-7 in these cells are up- regulated by the constitutive production of IFN. Consequently these data suggest that the analysis of the relative levels of IRF-7 in Namalwa cells may be a sensitive indicator how effectively these cells will be able to yield IFN-α when induced.

Furthermore we have found that in human fibroblasts, viral infection led to the expression of the IFN-β gene but not IFN-α genes. However, reconstitution of IRF-7 expression in these cells conferred the virus-mediated activation of 7 endogenous IFN-α genes from which expression of IFNA1 and A7 was most predominant. These data indicate that the lack of IFN-α gene expression in infected fibroblasts is due to the absence of IRF-7 in these cells rather than due to the presence of a transcription suppressor factor(s). By using a co-transfection assay, the expression of individual IFN-α reporter genes was determined in the

presence of over-expressed IRF-3 and the IRF-7 spliced variant IRF-7H. It was shown that while all the IFN-α promoters tested (A1, A2, A4 and A14) were activated by IRF-7, only A1 was activated by IRF-3.

Accordingly one aspect of the present invention relates to a method of controlling the level of Type I interferon expression in vitro, wherein the method comprises expressing the Type I interferon in an expression system which is capable of expressing interferon and which comprises one or more nucleotide sequences encoding at least one subtype of Type I interferon, wherein the level of interferon expression is controlled by controlling the level of IRF-7 or any functional analog thereof. Levels may be controlled by 'modulation' of levels of IRF-7 present to increase or decrease levels by whatever means, including addition or removal of IRF-7 from the expression system environment and/or up-regulation or down-regulation of the genes encoding IRF-7. The various means of modulation are described further below.

Type I interferons comprise interferon alpha (IFN-α), interferon beta (IFN-β) and interferon omega (IFN-ω). The preferred interferons are IFNα and β, with IFNα being the most preferred.

The expression system is any expression system capable of expressing interferon and includes yeast expression systems and bacterial expression systems such as those employed in E. Coli. Expression systems generally comprise vectors containing the gene or gene(s) of the desired product to be expressed and the necessary regulatory elements to enable the genes to be expressed in a host cell. However, preferred expression systems involve cells of lymphoid origin, especially B cell lines such as Namalwa, cells of fibroblast origin, especially human fibroblast cells, HeLa cells and CHO cells.

Type I interferons are the products of transcription of 13 functional genes of the interferon alpha (IFN-α) family or of the single interferon beta (IFN-β) or the single interferon omega (IFN-ω) genes.

The human interferon α gene family consists of 13 functional genes which are clustered on the short arm of chromosome 9 (Diaz, Sem. Virol. 6, 143-149,

1995). All of these genes show a high degree of similarity in the nucleotide sequences not only in the coding region, but also in the promoter region . The similarity can also be found between human and mouse interferon alpha Virus Responsive Elements (VRE). Differences in the levels of expression of individual interferon alpha subtypes in cells differs in a cell specific fashion and is due to the difference in the transcription activation of these genes (Bisat et al, Nucleic Acids Res. 16, 6067-6083, 1988).

As discussed above, the production of interferon -α is dependent on levels of IRF-7 in the cells and IRF-7 has been shown to activate interferon -α promoters and thus induce expression of the interferon -α genes which in turn induces production of interferon -α sub-types. Indeed, whilst IRF-7 activated all interferon -α promoters tested, IRF-3 only activated one interferon -α promoter (A1). However, IRF-3 can induce expression of the interferon -β gene.

It follows that by controlling the levels of IRF-3 the expression of interferon -β and the -α 1 subtype of interferon -α can be controlled, and by controlling the levels of IRF-7 the expression of interferon -α proteins can be controlled. For example, levels of endogenous IRF-7 can be increased in cells that either naturally do not express IRF-7, constitutively produce low levels of IRF-7, or are unresponsive to the inducers of IRF-7. Examples of increasing levels of IRF-7 include but are not limited to the following means:

Endogenous IRF-7 levels may be increased by treating cells with interferon-α. Other cytokines, chemokines or stimuli may have the same effect.

Another example is transfection of a cell with an expression vector that contains the nucleic acid sequence of IRF-7, any necessary regulatory elements and optionally a selectable marker. The marker allows selection of the transfectant and amplification of the inserted genes. If both IRF-7 and IRF-3 are required they may be co-transfected either in two separate vectors or in the same vector, including two genes linked on the same vector.

A further example is addition of IRF-7 peptide that is modified to be transferred to the cell nucleus, to the cell culture medium in which the host cells of the expression system are grown.

Another way of increasing levels of IRF-7 would be to decrease degradation of IRF-7 within the cell, by introducing agents that will interfere with degradation of IRF-7. Similarity, any agents that enhance the synthesis of IRF-7 could be introduced to increase the levels of IRF-7 within the expression system.

Generally, any method or agent capable of increasing the level of IRF-7 will be useful for enhancing the production of Type 1 interferons.

It will be appreciated that an increase in IRF-3 may be achieved in the same way. IRF-7 and IRF-3 may be increased either on their own or together.

It will be appreciated that IRF-7 and IRF-3 are preferably of human origin, they may also be of other origin, for example mouse. The production and purification of IRF-7 and IRF-3 are well known to the skilled person, however, examples of production and purification will be found in the following references; Au et al, J. Biol Clem. 273, 29210-29217 (1998); Marie et al, EMBO. J., 17, 6660-6669 (1998); Sato et al FEBS Lett. 441, 106-110 (1998); Juang et al Proc Natl. Acad. Sci USA 95, 9837-9842 (1998); Au et al Proc. Natl. Acad. Sci USA, 92, 11657-11661 (1995);

Under normal circumstances, IRF-3 and IRF-7 need to be activated by phosphorylation. It may therefore be advantageous to transfect cells with vectors that encode protein that is constitutively active and does not need to be activated by phosphorylation. Similarly, such constitutively active IRF-7 and/or IRF-3 may also be advantageously used in any of the other methods that require IRF-7 and/or IRF-3.

It will be appreciated that the present invention is not limited to IRF-7 and IRF-3, but will include any functional analogs thereof. Functional analogs will include but are not limited to pre-cursors of IRF-7 and IRF-3, variants and hybrids of IRF-7 and IRF-3 and any agents that will perform the same biological function within an

expression system as IRF-7 and IRF-3. The term 'functional analog' also includes nucleic acid, in particular DNA encoding the IRF-7 or IRF-3 genes which can be used to express IRF-7 or IRF-3.

In transient transfection assays, transactivation of individual subtypes of interferon -α VRE's, generally correlate with the ability of IRF-3 and IRF-7 to bind to the respective VREs (virus responsive elements within cells). It follows, that IRF-7 and IRF-3 analogs will include agents that are capable of transactivating individual subtypes of interferon -α VREs in transient transfection assays.

Although an increase in IRF-7 and/or IRF-3 will result in an enhancement in interferon production the effects of this enhancement will be greatly increased in the presence of an inducing agent , such as a virus. Thus, infection of cells with, virus,for example, Sendai virus in the presence of IRF-7 and/or IRF-3 will have a much greater effect on interferon production compared to the effect of either IRF-7 or virus on their own. Thus, for example, while IRF-7 increased the expression of the interferon A1 promoter and hence the interferon -α 1 protein only 2 fold, viral infection increased IRF-7 mediated stimulation 6 fold, indicating a synergistic activation by IRF-7 and virus. Similarly, expression of the -α interferon A2 promoter was increased 5 fold by IRF-7 alone but 16 fold after viral infection. The interferon A4 promoter showed a similar response to IRF-7 activation but in infected cells its expression was slightly lower (12 fold).Other inducing agents of interferon gene expression include but are not limited to double stranded RNA, such as ds RNA.

It follows that IRF-7, raises the level of all interferon -α subtypes but each of these subtypes is not raised to the same extent, but by an amount specific to that subtype. By knowing, how individual subtypes of interferon -α are affected by IRF-7, it is possible to control not only the overall level of interferon-α produced but also the level of each specific subtype. Moreover, by inserting and/or deleting the nucleotide sequences relating to specific subtypes the producion of interferon -α may be limited to just one specific subtype or to include a predetermined mixture of specific subtypes. This may be important in the treatment of certain conditions where one subtype or mixture of subtypes may provide better results

compared to other subtypes. Thus , for example, subtype 5 has been shown to be particularly good for the treatment of hepatitis.

The present invention also provides a novel method of treatment . Thus a second aspect of the present invention relates to a method of controlling the level of type I interferon expression in vivo . Specifically, IRF-7 or any functional analog thereof is administered to a mammal, preferably a human. The resulting raised levels of IRF-7 will increase production of interferon -α in response to viruses that under some circumstances, are not able to do so efficiently, for example in human hepatitis, papilloma and tumour viruses. The raised IRF-7 levels will also increase the producion of interferon -α in vivo in response to physiological and other stimuli, and so directly or indirectly , give beneficial results in various forms of cancer. Under other circumstances , they will stimulate the innate homeostatic mechanisms that resist virus infection of cells and oncogenic changes.

The invention therefore includes IRF-7 (and/or IRF-3) or functional analogs thereof for use in therapy. The invention further includes a nucleotide sequence encoding IRF-7 for use in therapy. Moreover, the invention provides the use of IRF-7 and/or IRF-3 and functional analogs thereof in the manufacture of a medicament for the treatment of a condition in which an increase in interferon expression is beneficial. Such conditions include human viruses such as hepatitis and herpes including human papilloma virus and cancers.

IRF-7 is a protein and therefore IRF-7 or any deriviative thereof may be administered by any method commonly employed for the administration of proteins.

The protein will usually be supplied as part of a sterile, pharmaceutically acceptable composition. This pharmaceutical composition may be in any suitable form, depending upon the desired method of administering it to a patient. It may be provided in unit dosage form and may be provided as part of a kit. Such a kit would normally (although not necessarily) include instructions for use.

Protein administrations are generally given parenterally, for example intravenously, intramuscularly or sub-cutaneously. The proteins are generally given by injection or by infusion. For this purpose protein is formulated in a pharmaceutical

composition containing a pharmaceutically acceptable carrier or diluent. Any appropriate carrier or diluent may be used, for example isotonic saline solution. Stabilizers may be added such as a metal chelator to avoid copper-induced cleavage. A suitable chelator would be EDTA, DTPA or sodium citrate.

They may be given orally or nasally by means of a spray, especially for treatment of respiratory disorders.

They may be formulated as creams or ointments, especially for use in treating skin disorders or virus induced skin lesions such as those caused by Herpes Simplex virus and Human Papilloma virus.

They may be formulated as drops, or the like, for administration to the eye, for use in treating disorders such as vernal conjunctivitis.

For injectable solutions, excipients which may be used include, for example, water, alcohols, polyols, glycerine, and vegetable oils.

The pharmaceutical compositions may contain preserving agents, solubilising agents, stabilising agents, wetting agents, emulsifiers, sweeteners, colourants, odourants, salts, buffers, coating agents or antioxidants. They may also contain other therapeutically active agents.

Suitable dosages of the substance of the present invention will vary, depending upon factors such as the disease or disorder to be treated, the route of administration and the age and weight of the individual to be treated. Without being bound by any particular dosages, it is believed that for instance for parenteral administration, a daily dosage of from 0.01 to 50 mg/kg of a binding agent of the present invention (usually present as part of a pharmaceutical composition as indicated above) may be suitable for treating a typical adult. More suitably the dose might be 0.05 to 10 mg/kg, such as 0.1 to 2 mg/kg.

This dosage may be repeated as often as appropriate. Typically administration may be 1 to 7 times a weeks. If side effects develop the amount and/or frequency of the dosage can be reduced.

A typical unit dose for incorporation into a pharmaceutical composition would thus be at least 1mg of binding agent, suitably 1 to 1000mg.

Alternatively, levels of IRF-7 may also be increased *in vivo* by administering the nucleotide sequence encoding IRF-7.

Suitable techniques for introducing a nucleic acid molecule or vector into a patient include sub-cutaneous application of the 'naked' nucleic acid in an appropriate vehicle. The nucleic acid may be present together with a pharmaceutically acceptable excipient, such as phosphate buffered saline (PBS). One technique involves particle bombardment (which is also known as 'gene gun' technology and is described in US Patent No. 5371015). Here inert particles (such as gold beads coated with a nucleic acid) are accelerated at speeds sufficient to enable them to penetrate a surface of a recipient (e.g. skin) by means of discharge under high pressure from a projecting device. (Particles coated with a nucleic acid molecule encoding IRF-7 or IRF-3 of the present invention are within the scope of the present invention, as are devices loaded with such particles.) Other methods of administering the nucleic acid directly to a recipient include ultrasound, electrical stimulation, electroporation and microseeding. Particularly preferred is the microseeding mode of delivery. This is described in US-5,697,901.

Nucleic acid molecules for use in the present invention may also be administered by means of specialised delivery vectors useful in gene therapy. Gene therapy approaches are discussed for example by Verme *et al*, Nature 1997, **389**:239-242. Both viral and non-viral systems can be used. Viral based systems include retroviral, lentiviral, adenoviral, adeno-associated viral, herpes viral and vaccinia-viral based systems. Non-viral based systems include direct administration of nucleic acids and liposome-based systems.

A nucleic acid sequence for use in the present invention may even be administered by means of transformed cells. Such cells include cells harvested from a subject. The nucleic acid molecules for use in the present invention can be introduced into such cells *in vitro* and the transformed cells can later be returned to the subject. The nucleic acid molecules need not be introduced into

the cells as vectors, since non-vector nucleic acid molecules can be introduced. Some such molecules may integrate into nucleic acid already present in a cell by homologous recombination events. A transformed cell may, if desired, be grown up *in vitro* and one or more of the resultant cells may be used in the present invention. Cells can be provided at an appropriate site in a patient by known surgical or microsurgical techniques (e.g. grafting, micro-injection, etc.)

Another way of treating a deficiency in the expression of a polypeptide comprises providing a patient with a DNA molecule that can be transcribed to provide the untranslated region of the present invention. This molecule can be provided in a manner to allow it to become operably linked with a sequence already present in the patient that encodes said polypeptide.

A further way of treating a deficiency in the expression of a polypeptide comprises providing a patient with an RNA molecule coding for said polypeptide, which RNA molecule is produceable by transcribing a DNA molecule for use in the present invention. The RNA molecule can then be translated *in vivo* to provide the polypeptide.

A still further way of treating a deficiency in the expression of a polypeptide, comprises providing a patient with the polypeptide, wherein the polypeptide has been produced using an expression system as described above.

The present invention is also useful in providing DNA vaccines. The direct injection of gene expression cassettes into a living host transforms a number of cells into factories for the production of the introduced gene products. Expression of these delivered genes has important immunological consequences and may result in the specific immune activation of the host against expressed antigens. Although vaccines produced by recombinant DNA technology are safer than traditional vaccines, which are based on attenuated or inactivated bacteria or viruses, they are often poorly immunogenic. Placing an untranslated region upstream of the coding sequence of a gene to be delivered in a DNA vaccine can significantly increase expression and can therefore increase immunogenicity. Due to the highly conserved mechanism of heat shock response an increase in polypeptide expression can be expected in every tissue where the gene is

delivered. DNA vaccines can be designed to prevent viral, bacterial and parasitic infections (e.g. diphtheria, malaria, leishmaniasis, toxoplasmosis, schistosomiasis, cryptosporidiosis, tuberculosis, HIV, HSV, influenza virus, hepatitis A, B and C), but can also be used for treating cancer, immune-related diseases or for contraceptive purposes. All of these applications are within the scope of the present invention.

When used in medicine, the nucleic acid molecules, vectors, polypeptides and cells discussed above will usually be in the form of a pharmaceutically acceptable composition. One or more pharmaceutically acceptable carriers may be present in such a composition. A pharmaceutical composition within the scope of the present invention may be adapted for administration by any appropriate route, for example by the oral (including buccal or sublingual), rectal, nasal, topical (including buccal, sublingual or transdermal), vaginal or parenteral (including subcutaneous, intramuscular, intravenous or intradermal) routes. Different drug delivery systems can be used to administer pharmaceutical compositions, depending upon the desired route of administration. Drug delivery systems are described, for example, by Langer (*Science* **249,** 1527–1533 (1991)) and by Illum and Davis (*Current Opinions in Biotechnology* **2**, 254–259 (1991)). In summary, it will be appreciated that the present invention can be used to manufacture medicaments for use in the treatment of one or more of the disorders discussed herein.

It will be appreciated that IRF-7 therapy can be administered in conjuction with IRF-3 therapy.

Conversely, it is sometimes beneficial if intra-nuclear levels of IRF-7 are reduced : cells will then produce less interferon -α. The resulting lower levels of IRF-7 will reduce the producion of interferon -α, with benficial results in circumstances where there is inappropriate, excessive or deleterious production of interferon -α in vivo, for example in rheumatoid arthritis, various manifestations of autoimmune disease and certain virus infections including late-stage AIDS. Methods for reducing IRF-7 levels include the use of chemicals that inhibit the intra-nuclear expression of IRF-7 or its functions; reduce IRF-7 gene expression in vivo ; or increase exretion of IRF-7 molecules from the nucleus or facilitate its

degradation. Substances that inhibit the functions of IRF-7 will have similar effects.

The invention is not limited to the enhanced production of Type I interferons, but also includes enhanced production of other early inflammatory genes and chemokines that are stimulated by virus infection and regulated by IRF factors such as RANTES, IL-8, and nitrous oxide synthetase.

Figures

Fig. 1. Distinct activation of interferon alpha(IFNA) promoters by IRF-3 and IRF-7 in transient transfection assay in HeLa cells.

HeLa cells were cotransfected with reporter plasmid containing the alkaline phosphatase cDNA under the control of t e IFNA1(Diagonals),IFNA4(White) and IFNA14(hached) promoter with , IRF-3 or IRF-7 expression plasmids (Juang et all PNAS 1998, Au et all JBC 1998) in the presence of a plasmid expressing beta galactosidase used as internal control. Transfection with IRF-1 expressing plasmid was carried out for comparison. When indicated cells were infected with Sendai virus 24 hrs post infection and the alkaline phosphatase levels in the medium were determined 16 hrs later.
(Diagonals) A1, (White) A4 and (Hached) A14

FIG. 2. Activation *of IFN-*α and *IFN-*β promoters by IRF-3 and IPF-7 in 2FTGH cells

(A) Reporter plasmids containing SAP cDNA under the control of *IFNA1(Diagonals), A2(Solid) ,A4(White) , A14(Hached)* and *B(Dotted)*VRE(500 ng) were co-transfected with 500 ng of plasmids expressing IRF-3 or IRF-7, and 50 ng of pCMV-β-galactosidase into 2FTGH cell were infected 24 h post-transfection with SeV for 16 h and levels of SAP in culture media were determined.

(B) Activation of *IFNA1(Diagonals)* and its mutants *A1 (4PM)(Solid), A1*

(NFκB)(White), A1 (Δ21)(Hached), and *B(Dotted)*VRE by IRF-3 and IRF-7.. Transfection and subsequent infection was carried out as described in *A above.*

(C). Alignment *IFNA1* and its mutants' VRE sequences. Nucleotides altered or inserted are boxed.

FIG.3. Binding of the recombinant GST- IRF-3 and GST -IRF-7 fusion proteins to the IFNA, VRE *A).* Electromobility shifi assay (EMSA) comparing the binding of GST-IRF-3 and GST-IRF-7 to the probes corresponding to the IFNA1, A2,A4 and A14 VRE. (B).Analysis of the GST-IRF-3 and GST-IRF_7 binding by DNA ase protection assay.

FIG.4.A. Expression of the endogenous IFN A genes in 2FTGH cells expressed ectopic IRF-7. A. Analysis of the relative levels of IRF-3 and IRF-7(Flag tagged) in transfected 2FTGH cells. Cells were collected 8 hrs post infection and the levels of IRF-3 and IRF-7 in cell lysates were determined by immune blotting with IRF-3 and Flag antibodies respectively.

B.Analysis of IFNA and IRF-7 mRNA. Total RNA was prepared from the infected transfected cells and parental cell line at 8h postinfection and the presence of IFNA and IRF-7 mRNA was determined by RT PCR, using primers corresponding to the conserved regions of the family of human IFNA genes. The amplified eDNA fragments were analyzed on agarose gells.

C.Interferon subtypes expressed in 2FTGH cells expressing IRF-7, were determined by cloning and sequencing of the amplified IFN-α cDNA. Fourty randomly selected clones were sequenced and analyzed.

Examples

Example 1: In transient transfection assays in HeLa cells, IRF-3 and IRF-7 differentially activate interferon alpha (IFN-α) promoters.

HeLa cells were co-transfected with a reporter plasmid containing a reporter gene, SAP (the cDNA of alkaline phosphatase) under the control of the IFNA1, or

IFNA4 or IFNA14 promoter, and with a plasmid expressing IRF-1 (used as a control), or IRF-3, or IRF-7 [Juang et al.(1988). Proc. Natl. Acad.Sci. U.S.A. **95** 9837-9842; Au et al. (1988). J. Biol. Chem **273** 29210-29217]. A beta-galactosidase plasmid served as an internal control, enabling differences in transfection efficiency to be normalized. 24 h after transfection, some of the cells were infected with Sendai virus (Sev) , while others remained uninfected. The levels of alkaline phosphatase in the medium were determined 16 h later. Results are shown in Fig. 1.

Cells transfected with any of the 3 promoter genes expressed only very small amounts of the SAP reporter, but on infection with Sendai virus, expression increased 6-fold in those transfected with IFNA1. Co-transfection with the IRF-1 plasmid had little effect on SAP expression, whether or not the cells were infected with Sendai virus. Co-transfection with the IRF-3 plasmid had little effect in uninfected cells, but in cells transfected with the IFNA1 promoter (but not with the IFNA4 or 14 promoters) and infected with Sendai virus, there was a 21-fold increase in expression of the SAP gene. Co-transfection with the IRF-7 plasmid and the IFNA1 promoter led to a 16-fold increase in phosphatase; if these cells were exposed to Sendai virus, there were dramatic increases of 72-fold, 36-fold and 27-fold in those co-transfected with the IFNA1 IFNA4 and IFNA14 promoters, respectively.

Example 2. Differences in the level of expression of various human IFN-α reflects different transcription activation of their genes.

Reporter plasmids (500 ng) containing SAP cDNA under the control of the Virus Responsive Elements (VRE) of IFNA1 (Diagonal), A2(Solid), A4 (White), A14 (Hached) and IFN-β, were co-transfected into 2FTGH human fibroblast cells together with 500 ng of plasmids expressing IRF-3 or IRF-7, and 50 ng of pCMV-β-galactosidase. Cells were infected 24 h post-transfection with SeV, and levels of SAP in the culture media were determined 16 h later. Results are shown in Fig. 2A.

2FTGH cells express IRF-3 constitutively, but do not express IRF-7. Virus infection stimulates the expression of the IFN-β gene but not of the IFN-α genes.

Transfection with the plasmids containing the various VRE for the different genes and the IFN-β gene did not lead to expression of the reporter SAP gene in uninfected cells, and virus infection only stimulated expression of the IFN-β gene. Co-transfection with IRF-3 increased 5-fold the constitutive expression of the IFN-β gene; virus infection further increased expression to 12-fold. Expression of the IFNB reporter gene was the same in virus infected and uninfected cells, possibly because there is already a high level of endogenous IRF-3 in these cells. Co-transfection with IRF-3 also led to a 2-fold increase in the IFNA1 reporter gene but there was no increase with the IFNA2, A4 or A14 genes.

In contrast, co-transfection with IRF-7 increased both the constitutive and the virus-induced expresssion of all the IFN VRE tested, but their relative expression differed. IRF-7 increased the expression of the IFNA1 reporter gene 2-fold, but in viruis-infected cells, the overall increase was 6-fold: this indicates synergistic activation by IRF-7 and virus. Expression of the IFNA2 SAP reporter gene was increased 5-fold by co-transfection with IRF-7, and to a total of 16-fold by virus infection; results with the IFNA4 gene were similar. Expression of the IFNA14 reporter gene was only marginally (2-fold) enhanced by IRF-7 and was not boosted by virus infection. Over-expression of IRF-7 increased both the constitutive and virus-stimulated expression of the IFNB reporter gene, but the increases were only additive. In summary, the data show that in this transient transfection system in 2FTGH fibroblasts, virus effectively stimulates the VRE only of IFN-β , and not of the various IFN-α tested. This correlates with the observed lack of expression of the endogenous IFN-α genes in infected fibroblast cells.

To determine more precisely which sequences within the VRE respond to IRF-7 activation in virus-infected cells, expression of various IFNAI VRE mutants inserted into the SAP reporter plasmid was examined. These mutants are shown in Fig.2C, with the VRE sequences aligned. Nucleotides altered or inserted are boxed. Mutant pA1 (Δ21)-SAP contains only sequences corresponding to the positive regulatory domains (PRD) 1 and III in IFNB VRE, which bind IRF-3. Mutant pAI pA1 (4 PM)-SAP contains 4 point mutations (G→A) in the GAAAG motifs.

In transient transfection assays (Fig.2B), mutant pAI (Δ21)-SAP was activated less efficiently by IRF-7 and virus infection than the wild-type IFNAI VRE, whilst the 4 point mutations in pA1 [4 PM]-SAP) completely abolished both the transcription activity of the IFNAI VRE and its response to IRF-7. In contrast, the insertion of the NFκB binding site into IFNAI VRE substantially enhanced the transcription activation of IFNAI VRE. This suggests that the insertion of NFKB site into IFNAI VRE converted it to a IFNB-like promoter.

Example 3 Binding of recombinant GST-IRF-3 and GST-IRF-7 fusion proteins to IFNA VREs.

We then investigated whether the differences between the capacity of IRF-3 or IRF-7 to activate the VRE of individual IFN-α subtypes correlate with their binding affinity to IFNA VRE.

Recombinant GST-IRF-3 and GST-IRF-7 fusion proteins were purified on a glutathione separose column, and the binding of each to the IFNAI, A2, A4 and A14 VRE was determined by EMSA.

GST alone did not bind to any of these probes (Fig. 3A). GST-IRF-3 bound effectively to the IFNAI probe resulting in the detection of a strong fast migrating band, and a weak slower migrating band, representing IRF-3 dimer. Binding of GST-IRF3 to the A2 and A4 probes led to the formation of weak fast moving bands, reflecting their much weaker binding. There was no detectable binding to the A14 VRE probe.

In contrast, GST-IRF-7 bound effectiently to both A I and A2 VRE and the formation of 3 protein-DNA complexes was detected. Presumably, these may be derived both from binding of IRF-7 dimers and binding to the multiple IRF binding sites on the VRE probe (Fig. 3B). Binding of GST-IRF-7 to the A4 probe led to the formation of only a slowly moving band, and no binding to the IFNA14 VRE could be detected. These binding specificities were confirmed by competition experiments. Similar specificities were confirmed for the binding of GST-IRF-7 (data not shown).

Since the Al VRE encompasses 86 bp, we used the DNase protection assay to determine the protected region through binding of GST-IRF-3 and GST-IRF-7 to *A1, A2* and *A14* probes (Fig. 3B). The binding of GST-IRF-7 as well as binding of the GST-IRF 7 DBD (aa 1-23 7) protected the region spanning nt - 1 03 to -67 in A1 VRE. The same region was protected by IRF-7 in *A2* VRE but no protection in this region was seen in *A14* VRE. Binding of GST-IRF-3 to A1 VRE protected the identical region, but no protection was detected upon incubation of GST-IRF-3 with A2 and A1 VRE. These data indicate that both GST-IRF-3 and GST-IRF-7 principally bind to the same region of A1 VRE. The lack of IRF-3 binding to IFN-α VRE other than A1 correlates with its inability to activate the corresponding IFN-α reporter gene. The - 103 to -67 nt region of A1 and A2 differs in nucleotides that are outside of the GAAAGC and GAAAGT binding domains. Which of these are critical for binding of IRF-3 remains to be determined.

Example 4. Reconstitution of endogenous IFNA gene expression in IRF-7 transfected human fibroblasts.

Data in the previous examples indicate that IRF-7 has a critical role in the activation of IFN-α VRE in infected cells. To see if virus is unable to stimulate the expression of IFN-α genes in 2FTGH fibroblasts because these do not express IRF-7, cells were transfected with IRF-3 and IRF-7 expressing plasmids and infected with Sendai virus 24 h later. The cells were harvested 8 h after infection and the presence of IRF-3 and IRF-7 in cell lysates was determined by Western blot analysis with anti-IRF-3 and anti-IRF7 antibodies, respectively (Fig. 4A). It can be seen that low levels of IRF-3 but no IRF-7 could be detected in untransfected cells. In cells transfected with IRF- 3 or IRF-7 expressing plasmids, the respective proteins could be detected both before and after viral infection. No decreases in the levels of IRF-3 were detected in infected cells, but the relative levels of IRF-7 were lower in infected than in uninfected cells.

The relative levels of IFN-α mRNAs in total RNA prepared from the infected and IRF-7 transfected cells were determined by RT PCR (Fig.4B). RNA from infected and uninfected 2FTGH cells, and also RNA isolated from infected cells transfected with a IRF-3 expressing vector were isolated 8 h post infection. The

presence of IRF-7 mRNA was detected only in transfected cells. Similarly, IFN-α mRNA could be detected only in cells that were expressing IRF-7, but not in infected parental cells or cells transfected with the IRF-3 expressing plasmid. The culture media from the infected cells were tested for human IFN-α by antiviral assay on bovine tracheal cells (which are sensitive to IFN-α but not to IFN-β). Interferon activity (80 U/ml) was detected only in media from the IRF-7 tansfected cells , but not in media from any of the other infected cells (Fig. 4B and Table 2). Since the transfection efficiency in 2FTGH cells is only about 25%, these levels of interferon underestimate the true values. However, neither IFN-α mRNA nor biologically active IFN-β could be detected in cells over-expressing IRF-3. Three clones of 2FTGH cells permanently transfectd with IRF-7 were infected with Sendai virus. Table 3 shows the amounts of biologically active IFN-α 1 formed as detected in an antiviral assay and the proportion of the subclones obtained from these that expressed the IFN-α 1 subtype.

It has been reported that in immortalized mouse embryo fibroblast cells, virus-induced expressions of non-IFNA4 genes was detected after the induction of IFN-α4 and IFN-β, which stimulated the expression of IRF-7. In contrast, in human 2FTGH cells, IFN treatment did not induce the expression of IRF-7, or prime virus-mediated induction of IFN-α genes (unpublished data).

The presence of IFN-α mRNA in RF-7 transfected 2FTGI-1 cells was detected by amplification with primers corresponding to the regions of IFN-α genes that are conserved in all IFN-α subtypes. In order to determine which IFN-α subtypes are expressed in IRF-7 transfected fibroblasts cells, the PCR-amplified cDNA fragment was cloned into the pBluescript KS II+ plasmid, and cDNA inserts isolated from 40 randomly selected clones were sequenced. DNA sequences obtained were compared with sequences of individual IFN-α genes present in GenBank. Expression of 7 IFNA subtypes were identified (Table 1), with a significant difference in the relative frequency of the individual IFN-α cDNA subtypes: IFNA1 and IFNA7 transcripts were most abundant (40% and 35%, respectively); IFNA4 gene was expressed at higher level (10%) than IFN10 and IFNA17 (5%); IFNA2 and IFNA14 were only marginally expressed (2.5%). In the transient expression assay in 2FTGH cells, IRF-7 was the most efficient activator of IFNA2 VRE. However, in the cells expressing ectopic IRF-7, the endogenous

IFNA2 gene was induced only with low ftequency, indicating that additional factor(s) may affect transcription of IFNA genes from the chromatin template. Altogether, these data indicate that in human fibroblast cells, virus-mediated induction of all IFN-α genes depends on the presence of IRF-7.

Three clones of 2FTGH cells permanently transfected with IRF-7 were infected with Sendai virus. Table 3 shows the amounts of biologically active IFN-α1 formed, as detecteded in an antiviral assay, and the proportion of the subdones obtained from these that expressed the IFNA1 subtype.

It has been reported that in immortalized mouse embryo fibroblast cells, virus-induced expression of non-IFNA4 genes was detected after the induction of IFN-α4 and IFN-β, which stimulated the expression of IRF-7. In contrast, in human 2FTGH cells, IFN treatment neither induced the expression of IRF-7 nor primed virus mediated induction of IFN-α genes (30; unpublished data).

The presence of IFN-α mRNA in IRF-7 transfected 2FTGH cells was detected by amplification with primers corresponding to the regions of IFN-α genes that are conserved in all IFN-α subtypes. In order to determine which IFN-α subtypes are expressed in IRF-7 transfected fibroblasts cells, we cloned the PCR-amplified cDNA fragment into the pBluescript KS II+ plasmid, and cDNA inserts isolated from 40 randomly selected clones were sequenced. DNA sequences obtained were compared with sequences of individual IFN-α genes present in GenBank. Expression of 7 IFN-α subtypes was identified, with a significant difference in the relative frequency of the individual IFN-α cDNA subtypes (Table 1). IFN-α1 and IFNA7 transcripts were most abundant (40% and 35%, respectively). IFNA4 gene was expressed at higher level (10%) than IFNA10 and IFNA17 (5%). IFNA2 and IFNA14 were only marginally expressed (2.5%). In the transient expression assay in 2FTGH cells, IRF-7 activated IFNA2 VRE most efficiently. However, in the cells expressing ectopic IRF-7, the endogenous IFNA2 gene was induced only with low frequency indicating that additional factor(s) may affect transcription of IFNA genes from the chromatin template. Altogether, these data indicate that in human fibroblast cells, virus mediated induction of all IFNA genes depends on the presence of IRF-7.

Example 5. Amounts of IFN-α formed by two clones of human lymphoblastoid cells.

Two clones of cells of human lymphoblastoid cells of the Burkiff lymphoma Namalwa line, B21GF8 and B31, were obtained from Glaxo Wellcome Beckenham, UK; these formed respectively relatively high and relatively low levels of biologically active IFN-α spontaneously, and also when stimulated with Sendai virus (Table 4). The clones expressed essentially the same amounts of IRF-3, as determined by Western blot hybridisation on the protein formed; however, expression of IRF-7. as determined by RT/PCR, was significantly greater in the high interferon producing clone. Thus in these human lymphoblastoid cells, production of IFN-α was again correlated with the expression of IRF-7.

Table 1. ***IFN-α*** **subtypes stimulated by viral infection in 2FTGH cells transiently expressing ectopic IRF-7.**

IFN-α	*#positives/40* clones	%
A1	**16**	**40**
A2	**1**	**2.5**
A4	**4**	**10**
A7	**14**	**35**
A10	**2**	**5**
A14	**1**	**2.5**
A17	**2**	**5**

2FTGH cells were transfected with IRF-7 expressing plasmid and 24 hrs later infected with Sendai virus for 16 Hrs.Total RNA was isolated and transcribed with oligo dT primers to cDNA. IFN-α specific transcripts were amplified by PCR using the conserved primers as described in Fig.4 Amplified fragments were cloned into Blue script plasmid, 40 clones were randomly selected and their DNA inserts sequenced and compared with IFNA sequences present in the Gene Bank data base.

Table 2. Induction of biologically active IFN-α in 2FTGH clones permanetly transfected with IRF-7.

Clone #	Sendai virus	IFN-α titres units/ml	
cl 1	0	<1	
	+	21	
cl 2	0	<1	
	+	60	
cl 3	0	<1	
	+	864	*782
cl 4	0	<1	
	+	160	
cl 5	0	<1	
	+	44	
cl 6	0	<1	
	+	20	
cl 7	0	<1	
	+	883	*1555
cl 8	0	<1	
	+	32	
cl 9	0	<1	
	+	128	*2048

+ Interferon assays were carried out on bovine cells that detect only IFN -α;titers are expressed in international units using recombinant IFNA2 as a standard control

* Titers were carried out after recloning of the clones from 24 well panel to 6 well panels

Table 3. Frequency of virus induced expression of IFNAI subtype in 2FTGH-IRF-7+ cells

clone #	IFNA u/mi	# of IFNA1 clones/ # of tested clones	% of IFNA1
cl 3	782	13/24	54
cl 7	1555	8/12	67
cl 9	2048	7/14	50

Table 4. Expression of IRF-3 and IRF-7 in Namalwa cells that produce high and low levels of IFNA upon stimulation with Sendai virus.

Namalwa cells	Spontaneous IFN	Sendai Induced IFN	IRF-3+	IRF-7*
B21 F8	20u/ml	5000-6000 u/mi	+++	++
B3OB1	2u/ml	100-200 u/mi	+++	+/-

+expression determined on protein levels by Western blot hybridisation
*expression determined on RNA level by RT/PCR

CLAIMS

1. A method of controlling the level of Type 1 interferon expression in an expression system capable of expressing at least one subtype of Type 1 interferon wherein the level of interferon expression is controlled by modulating the level of IRF-7 or a functional analogue thereof.

2. A method according to claim 1 wherein the Type 1 interferon is interferon-α.

3. A method according to either claim 1 or claim 2 wherein the expression system involves cells of human lymphoid or fibroblast origin.

4. A method according to any of the preceding claims wherein the level of interferon expression is controlled by also modulating the level of expression of IRF-3 or a functional analogue thereof.

5. A method according to any of the preceding claims wherein the modulation is to increase levels of IRF-7.

6. A method according to Claim 5 wherein the increase in levels of IRF-7 are achieved by treatment of the cells of the expression system with a cytokine, chemokine, or by addition of IRF-7 to the cell culture medium.

7. A method according to Claim 5 wherein the increase in levels of IRF-7 are achieved by transfection of the cells of the expression system with an expression vector capable of expressing IRF-7.

8. A method according to any of Claim 5-7 in which the cells are additionally treated with an inducing agent.

9. IRF-7 or a functional analogue thereof for use in therapy.

10. A nucleotide sequence encoding IRF-7 or a functional analogue thereof for use in therapy.

11. Use of IRF-7 or a functional analogue thereof in the manufacture of a medicament for the treatment of a condition in which an increase in interferon-α is beneficial.

12. Use according to Claim 11 for the treatment of human viral infections or cancer.

13. A pharmaceutical composition comprising IRF-7 or a functional analogue thereof and a pharmaceutically acceptable carrier.

14. A pharmaceutical composition according to Claim 13 wherein the functional analogue of IRF-7 is a nucleotide sequence encoding IRF-7.

Figure 1

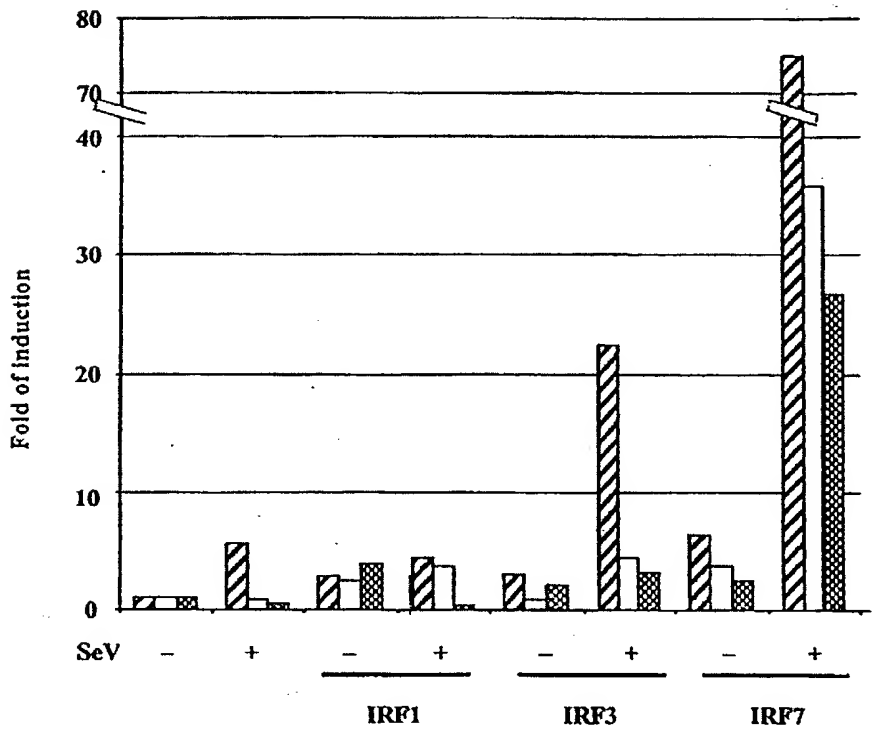
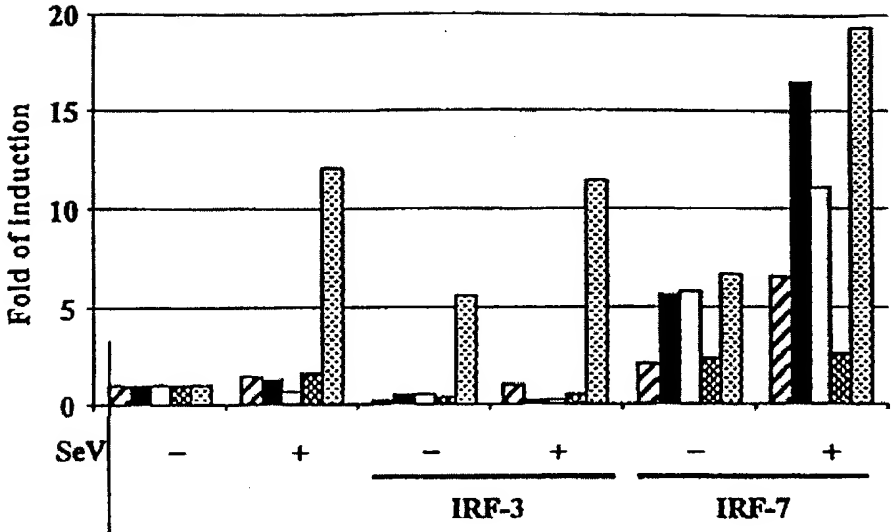


Figure 2.

A.



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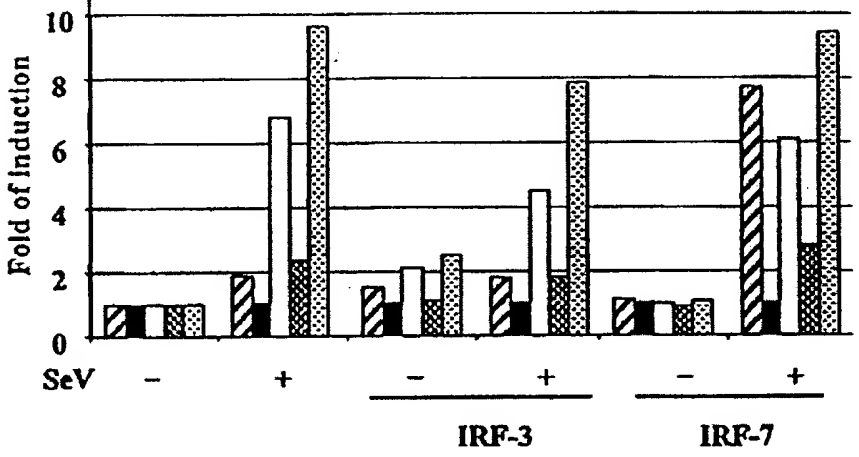


C.

```
A1          GAGTGCATGA AGGAAAGCAA AAACAGAAAT GGAAAGTGG-
A1 (4PM)    GAGTGCATGA AGGAAAGTAA AAACAGAAAT GGAAATTGG-
A1 (NFκB)   GAGTGCATGA AGGAAAGCAA AAACAGAAAT GGAAAGTGGC
A1 (Δ21)    GAGTGCATGA AGGAAAGCAA AAACAGAAAT GGAAAGT---

A1          -----CCCAG AAGCATTAAG AAAG
A1 (4PM)    -----CCCAA AAGCATTAAG AAAA
A1 (NFκB)   AGATTCCCAG AAGCATTAAG AAAG
A1 (Δ21)    ---------- ---------- ----
```

Figure 3

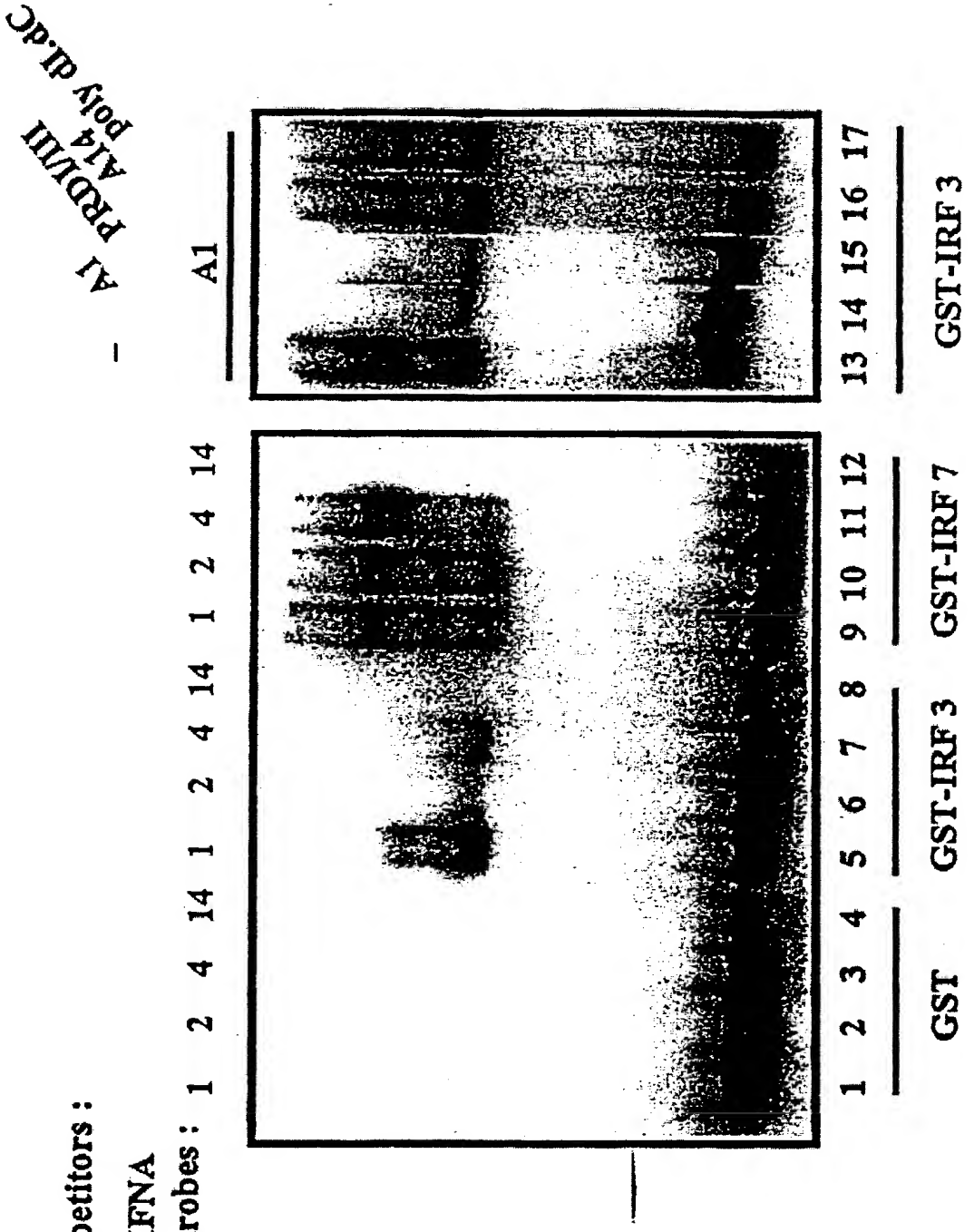

A.
competitors :
IFNA
probes : 1 2 4 14 1 2 4 14 1 2 4 14
A1
PRDI/III
A14
poly dI.dC
1 2 3 4 5 6 7 8 9 10 11 12
13 14 15 16 17
GST
GST-IRF 3
GST-IRF 7
GST-IRF 3

Figure 4

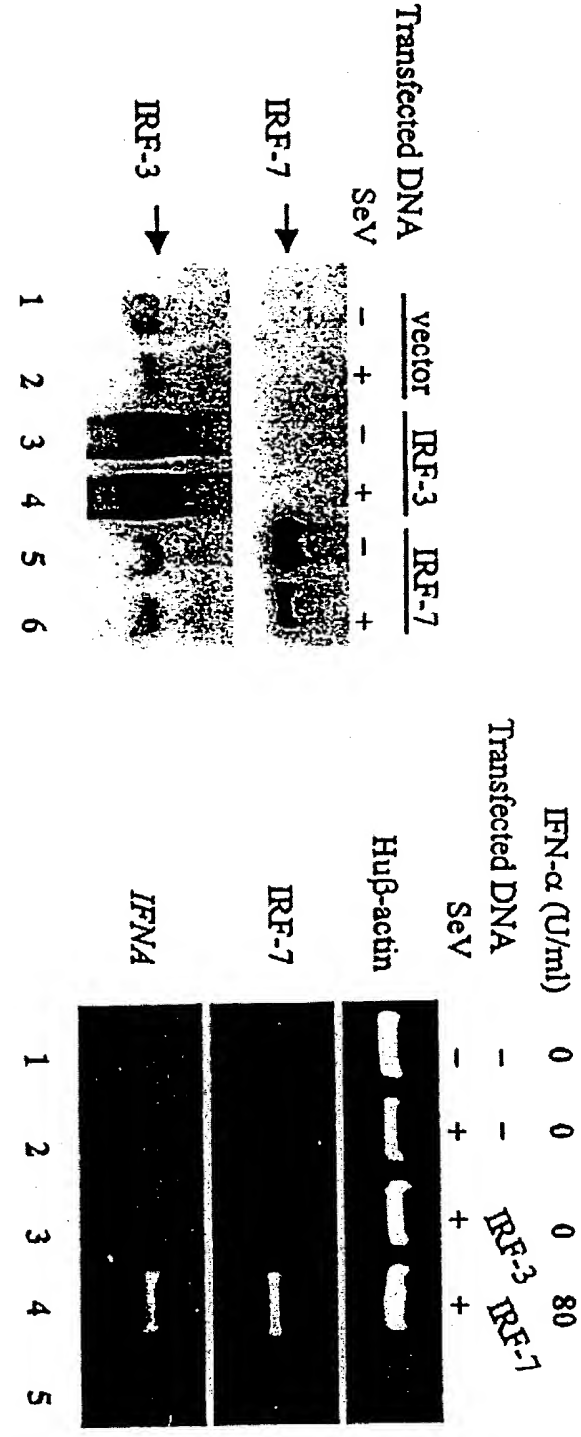
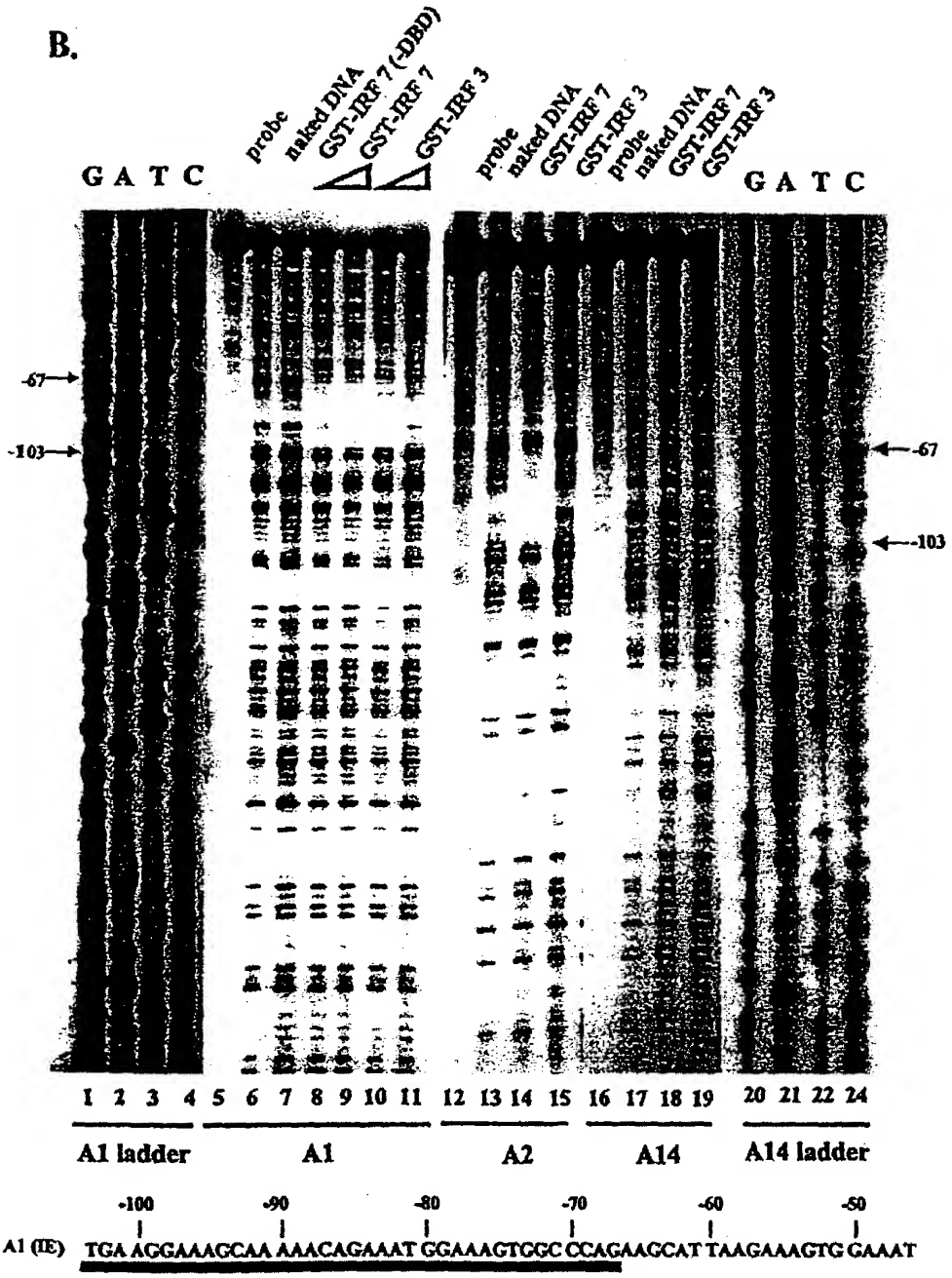

A
Transfected DNA
vector
IRF-3
IRF-7
SeV
- + - + - +
IRF-7
IRF-3
1 2 3 4 5 6
B
IFN-α (U/ml) 0 0 0 80
Transfected DNA
SeV - - + +
IRF-3 + +
IRF-7 + +
Huβ-actin
IRF-7
IFNA
1 2 3 4 5

Figure 4.


B.
probe
naked DNA
GST-IRF 7 (-DBD)
GST-IRF 7
GST-IRF 3
G A T C
-67
-103
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 24
A1 ladder
A1
A2
A14
A14 ladder
-100 -90 -80 -70 -60 -50
A1 (IE) TGA AGGAAAGCAA AAACAGAAAT GGAAAGTGGC CCAGAAGCAT TAAGAAAGTG GAAAT